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IMMUNOHISTOCHEMICAL EXPRESSION OF CANCER STEM CELL MARKER (ALDH1) IN A PROSTATE CANCER

Hadeel Ismail Mohsen*, Basim S. H. Ahmed and Adnan I. Ali

Al-Mustansyria University College of Medicine Baghdad/ Iraq.

***Corresponding Author: Hadeel Ismail Mohsen**

Al-Mustansyria University College of Medicine Baghdad/ Iraq.

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ABSTRACT

Background: Cancer stem cells were identified in several cancers, including PCa, and have been proposed to explain the metastatic capacity, recurrence, and resistance to hormone, radio and chemotherapy in established cell lines from PCa origin, particularly from metastasis. Enzymatic assays and immunohistochemistry have identified several proteins and surface markers that are expressed by CSCs and may be used to identify them in patients. **Aim of the study:** To assess the immunohistochemical (IHC) expression patterns of the ALDHmarker in PCa, BPH and in apparently normal looking prostate tissue. **Methods:** A cross-sectional Study carried out at department of histopathology and forensic medicine at Al-Mustansirya medical college and teaching lab of Al-Yarmook Teaching Hospital during a period of one year from (1st of august 2017 to 30th of July 2018). It involved 90 specimens; and these specimens were divided into the following: 60 of them were collected retrospectively depending on the archived files of patients diagnosed either with BPH (30 specimens) or with PCa (30 specimens) in the years 2016 and 2017 in Al-Yarmook Teaching Hospital and in the private labs. The other 30 specimens of normal prostatic tissue were collected prospectively from autopsy presented in forensic medicine directorate during the first three months of 2018. All patients diagnosed with BPH or PCa during 2016 and 2017 were included without any exclusion. Positive control: Human gallbladder tissue for ALDH marker with each run. **Results:** The mean age of the patients was 73.71 ± 8.15 years. The highest proportion of specimens of PCa (Group A) was graded ≤ 7 by Gleason Grading System (53.3%). There were significant associations ($P < 0.05$) between all these CSC marker results and prevalence of PCa. Positive ALDH marker result was significantly associated (P=0.024) with high Gleason Grading System. **Conclusion:** ALDH1 is significantly expressed in prostate carcinomas, and more likely so in cases with Gleason grading system 8.

KEYWORDS: PCa, CSC markers, BPH, Iraq.

INTRODUCTION

Prostate cancer (PCa) is the second leading cause of male malignancy death throughout the world.[1] The age is the strongest risk chance of getting prostate cancer, where the incidence of prostate cancer is rising rapidly after age 50.[2]

Incidence of prostate cancer highest in Scandinavian countries (22 cases / 100.000 population), this results may be inversely related to the ultraviolet light exposure as the incidence increases the farther one lives from the quarter.[3]

About (70-80%) of the prostate carcinoma arise in peripheral glands and early lesions appear as defined masses just beneath the capsular of the prostate. Most prostatic carcinoma is adenocarcinoma which composed of small glands (make prostatic fluid) that infiltrate to adjacent stroma.[4]

Biopsy play a role in diagnosis if cancer is suspected, a biopsy is offered expediently which is an outpatient procedure and require antibiotic cover to prevent infection.[5] Benign prostatic hyperplasia is a clinical condition where lower urinary tract symptoms are caused by both a physically obstructing prostate as well as tight smooth muscles around the bladder outlet.[6]

Prevalence of BPH increases from 8% in 31 to 40-year-old men, to 40 – 50% in 51 to 60-year-old men, and to over 80% in men older than 80 years and Observational studies from the US, Europe and Asia have established that older age is a risk factor for BPH onset and clinical progression by a number of different metrics.[6]

In recent years, cancer stem cells (CSCs) were identified in several cancers, including PCa, and have been proposed to explain the metastatic capacity, recurrence, and resistance to hormone, radio and chemotherapy in established cell lines from PCa origin, particularly from metastasis.[7]

CSCs have been identified and isolated using different approaches, such as flow cytometry, magnetic-associated cell sorting (MACS), the ability of differential cloning and sphere growing under non-adherent culture conditions.[8]

The classical concept of cancer pathogenesis stipulates that tumors arise from stepwise accumulation of multiple mutations in mature somatic cells and that all cells in a cancer share similar molecular aberrations. The cancer stem cell (CSC) theory is an alternative, fairly new paradigm and hypothesizes that distinct clones of cancer cells result from abnormalities that occur within specific cell types.[9]

In this theory, cells in a tumor do not have the same malignant potential but rather there are small clones of CSCs with self-renewal capabilities[10], high proliferative potential and pluripotency status that are responsible for tumor initiation, propagation and metastases.[11]

Enzymatic assays and immunohistochemistry have identified several proteins and surface markers that are expressed by CSCs and may be used to identify them in patients.[12]

MATERIAL AND METHODS

This is a cross-sectional Study carried out at department of histopathology and forensic medicine at Al-Mustansirya medical college and teaching lab of Al-Yarmook Teaching Hospital during a period of one year from (1st of august 2017 to 30th of July 2018).

The total number of 90 spacemen were collected from tow governorate places as Al-Yarmook Teaching Hospital and forensic medicine directorate in addition to private labs in Baghdad.

The study was conducted on human prostatic tissue specimens obtained from patients attending the hospital and labs after surgical removal of prostate gland and from autopsy presented in forensic medicine directorate.

This study involved 90 specimens; and these specimens were divided into the following: 60 of them were collected retrospectively depending on the archived files of patients diagnosed either with BPH (30 specimens) or with PCa (30 specimens) in the years 2016 and 2017 in Al-Yarmook Teaching Hospital and in the private labs. The other 30 specimens of normal prostatic tissue were collected prospectively from autopsy presented in forensic medicine directorate during the first three months of 2018.

All patients diagnosed with BPH or PCa during 2016 and 2017 were included without any exclusion.

- ✓ Positive control: human placenta tissue for EZH2 marker with each run
- ✓ Human gallbladder tissue for ALDH marker with each run
- ✓ Human bronchial tissue for SOX2 marker with each run
- ✓ Negative control: It was done by deleting the primary antibody and adding antibody diluent alone in the same slide and follows the same steps in histoimmunostaining.

Patients were divided into three groups according to pathological diagnosis of freshly prepared H & E stained slide:

- Group A: Included 30 patients proved to have PCa.
- Group B: Included 30 patients proved to have BPH.

All the sample of those two groups (A&B) were formalin fixed paraffin impeded blocks.

- Group C: Included 30 patients to have healthy looking normal prostatic tissue.

Preparation of tissue section (group C)

Bancroft, 2008 ordered procedure were used to prepared paraffin embedded tissue blocks of prostate tissue sample (group C) in the following order, Fixation, dehydration, clearing, impregnation, embedding, sectioning, de-waxing, hydration, followed by staining and mounting.

Immunohistochemistry staining
Material
1. Primary antibody kit
The primary antibody kits (sources and dilutions)

Primary Antibody	Source	Type	Dilution
SOX-2	Santa Cruz Code: sc-365964	Mouse monoclonal antibody	1:50
EZH2	Santa Cruz Code: sc-137255	Mouse monoclonal antibody	1:50
ALDH1A1	Santa Cruz Code: sc-374149	Mouse monoclonal antibody	1:50

2. Secondary detection kit
Secondary detection system from Santa Cruz

Materials	Quality	Description
Peroxidase block	100 ml	3% hydrogen peroxide water biotin labeled affinity isolated
m-IgGk BP-HRP	200 µg in 0.5 ml	Mouse igg kappa binding protein conjugated to horse dish peroxidase that diluted in protein block 1:100
Substrate DAB buffer	100 ml	Imidazole HCl buffer PH 7.5 containing hydrogen peroxide and antimicrobial.
DAB chromogen	10 ml	3-3 diaminobenzidine solution

2. **Primary antibody diluent from Santa cruse**
3. **Target retrieval solution PH 6 (sodium citrate buffer)**
4. **Phosphate buffer solution (dako)**
5. **Ethanol alcohol (absolute)**
6. **Xylene**
7. **Distil water**
8. **Aqueous moating media**

- **Examination:** The slides were exanimated under light microscope.

IHC and quantitative scoring method
The cells were scored as positive or negative staining depending on the presence of distinct brown cytoplasmic or nuclear staining. All tissue sections of the three groups were correlated with age, Gleason Score and bad prognostic sign.

The slides were examined with low power microscopy 10x to determine the regions of highest staining, if they show no staining at low power re-examination was done by high power 40x to determine area of weak staining, five fields of each slide were examined and scored semi quantitatively by calculating the proportion of positive stained cells over the total number of malignant cells (%).

ALDH: Slides were reviewed to evaluate staining expression of the marker under light microscope it shows cytoplasmic staining.

The percentage of cells staining (0 – 100%) was recorded for each case and scored as follows:
0–33%, score 1
34–66%, score 2
≥ 67%, score 3

The intensity of staining
0, none
1, mild
2, moderate
3, strong

Statistical Analysis
The data analyzed using Statistical Package for Social Sciences (SPSS) version 25. The data presented as mean, standard deviation and ranges. Categorical data presented by frequencies and percentages. Pearson's Chi–square test was used to assess statistical association between different associated variables. A level of P – value less than 0.05 was considered significant.

Ethical approval
Ethical approval for the study was obtained from the ethical committee of Al-Yarmook Teaching Hospital and forensic medicine directorate. The pathological diagnosis of BPH and PCa was confirmed by reviewing a freshly prepared hematoxylin and eosin stained slides.

RESULTS
The total number of study patients was 90 divided into three groups:

- Group A: Included 30 patients diagnosed with Pca.
- Group B: Included 30 patients diagnosed with BPH.
- Group C: Included 30 healthy patients (Controls).

Cancer Stem Cell Markers (CSC)
Table 4.2 shows the distribution of study groups by CSC marker(ALDH) results. In this study, ALDH was positive in 60% of specimens of Group A (PCa), 36.7% of specimens of group B (BPH), and 20% of specimens of group C (Control).

Table: Distribution of study groups by CSC marker results.

Cancer Stem Cell Marker Result	Group A n= 30 (%)	Group B n= 30 (%)	Group C n= 30 (%)	Total n= 90 (%)
ALDH				
Positive	18 (60.0)	11 (36.7)	6 (20.0)	35 (38.9)
Negative	12 (40.0)	19 (63.3)	24 (80.0)	55 (61.1)

Scoring and Intensity of markers in study groups

ALDH Marker

Scoring of ALDH marker in study groups is shown in figure (). We noticed that in Group A, ALDH marker scored (2) in 61.1% of the specimens, while it scored (1) in 72.2% of specimens of group B. It was equally scored (1) and (2) in specimens of group C (50% for each score).

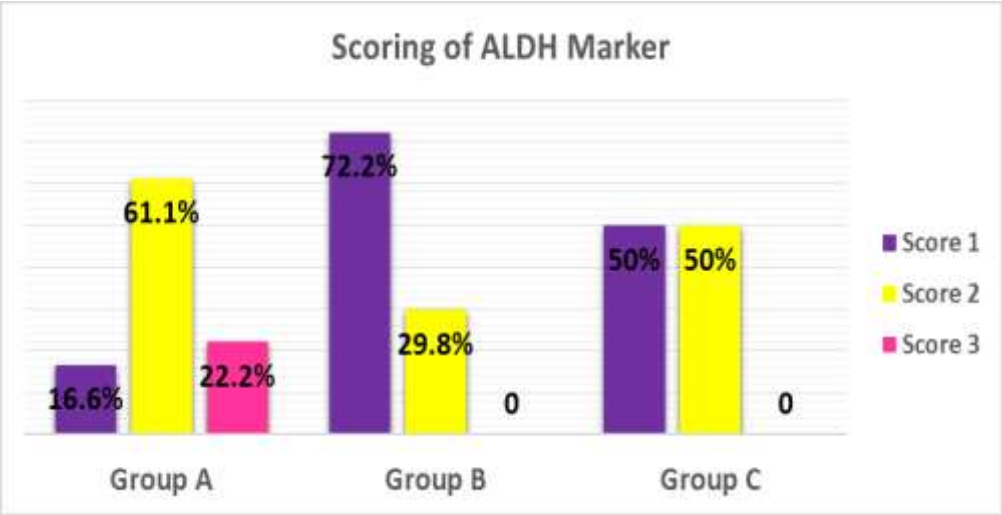

Figure 4.1: Scoring of ALDH marker in study groups.

Figure shows the intensity of ALDH marker in study groups. Moderate intensity was the most common intensity of ALDH marker in group A and B (61.1% and 81.8% respectively), while in group C, weak intensity represented the most prevalent intensity (66.7%).

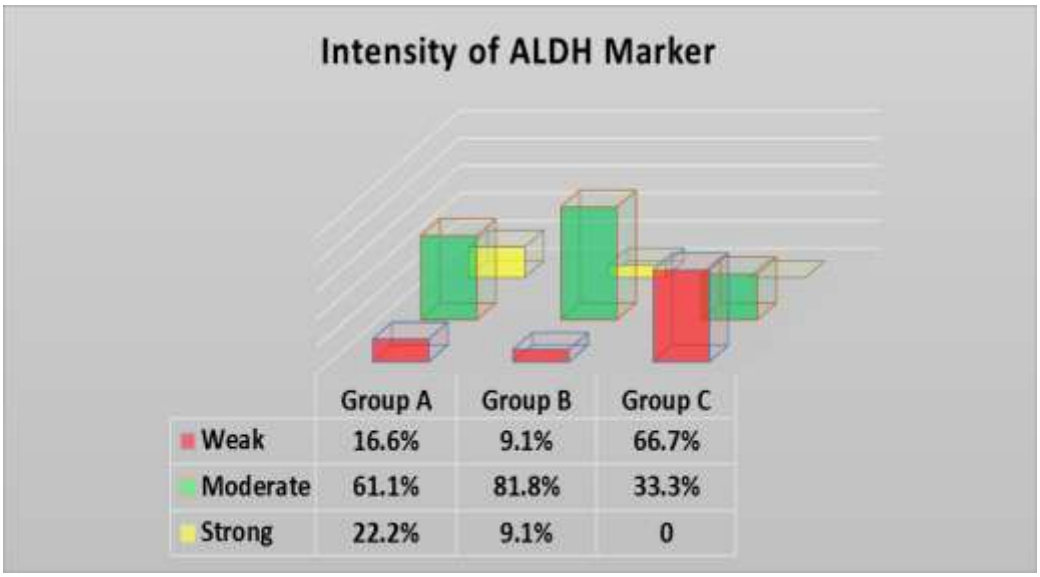

Figure 4.2: Intensity of ALDH marker in study groups.

Gleason Grading System of PCa

The distribution of study patients of Group A by Gleason Grading System is shown in figure (4.3). The highest proportion of specimens of PCa (Group A) was graded ≤ 7 by Gleason Grading System (53.3%).

Figure 4.3: Distribution of study patients of Group A by Gleason Grading System.

The association between Gleason Grading System and CSC marker result is shown in table (4.5). In this study, 55.6% of positive ALDH marker results were seen in grade between 8 – 9 with a significant association between Gleason Grading System and ALDH marker result (P=0.024).

Table 4.5: Association between Gleason Grading System and CSC marker result.

Variable	Gleason Grading System			Total (%) n= 30	P- value
	≤ 7 n= 16	8 – 9 n= 12	> 9 n= 2		
ALDH					
Positive	6 (33.3)	10 (55.6)	2 (11.1)	18 (60.0)	**0.024**
Negative	10 (83.3)	2 (16.7)	0 (0)	12 (40.0)	

CSC Marker and age group
In PCa (Group A)

The association between CSC marker result and age group in PCa specimens (Group A) is shown in table (4.6) It was clear that there was no significant association (P ≥ 0.05) between ALDH marker result and age group in PCa specimens.

Table 4.6: Association between CSC markers result and age group in PCa specimens (Group A).

CSC Marker	Age Group (Years)				Total (%) n= 30	P- value
	< 60 n= 6	60 – 69 n= 7	70 – 79 n= 13	≥ 80 n= 4		
ALDH						
Positive	2 (11.1)	5 (27.8)	8 (44.4)	3 (16.7)	18 (60.0)	**0.466**
Negative	4 (33.3)	2 (16.7)	5 (41.7)	1 (8.3)	12 (40.0)	

4.5.2. In BPH (Group B)

The association between CSC marker result and age group in BPH specimens (Group B) is shown in table (4.7). There was no significant association (P ≥ 0.05) between CSC marker result and age group in BPH specimens.

Table 4.7: Association between CSC markers result and age group in BPH specimens (Group B).

CSC Marker	Age Group (Years)				Total (%) n= 30	P- value
	< 60 n= 3	60 – 69 n= 11	70 – 79 n= 9	≥ 80 n= 7		
ALDH						
Positive	0 (0)	6 (54.5)	3 (27.3)	2 (18.2)	11 (36.7)	**0.321**
Negative	3 (15.8)	5 (26.3)	6 (31.6)	5 (26.3)	19 (63.3)	

4.5.3. In control (Group C)

Table 4.8 shows the association between CSC marker result and age group in control specimens (Group C). Half of patients with positive ALDH result were aged between 60 – 69 years with a significant association between age group and ALDH marker result ($P < 0.05$).

Table 4.8: Association between CSC markers result and age group in control's specimens (Group C).

CSC Marker	Age Group (Years)					Total (%) n= 30	P- value
	< 20 n= 2	20 – 29 n= 19	30 – 39 n= 4	40 – 49 n= 1	60 - 69 n= 4		
ALDH							
Positive	0 (0)	0 (0)	2 (33.3)	1 (16.7)	3 (50.0)	6 (20.0)	**0.001**
Negative	2 (8.3)	19 (79.2)	2 (8.3)	0 (0)	1 (4.2)	24 (80.0)	

DISCUSSION

The cancer stem cell (CSC) theory is an alternative, fairly new hypothesises that distinct clones of cancer cells result from abnormalities occurred within specific cell.[13] In this theory, there are small clones of CSCs with self-renewal capabilities, high proliferative potential and pluripotency status that are responsible for tumor initiation, propagation and metastases.[14] Enzymatic assays and immunohistochemistry have identified many proteins and surface markers that are expressed by CSCs and may identify them in patients.[15] Aldehyde dehydrogenase 1A1 (ALDH1) is an intracellular enzyme that oxidizes retinol to retinoic acid and there is an evidence that ALDH1 is overexpressed in CSCs of various organs as hematopoietic, breast, colorectal and prostate.[16] ALDH1 showed higher expression in renal cell carcinoma tissues than normal renal tissues and it is associated with clinicopathological variables (grade, size, stage, renal vein invasion, and perinephric fat invasion). This may reflect the role of ALDH1 in disease progression and poor prognosis of RCC.[17] The total number of patients included in the current study were 90 divided into three groups: 30 patients with Pca (Group A), 30 patients with BPH (Group B) and 30 healthy patients (Group C).

5.1. Cancer Stem Cell Markers (CSC)

5.1.1 ALDH Marker

About two third of patients (60%) with PCa (Group A), nearly one third (36.7%) of those with BPH (Group B) and only 20% of control group (Group C) had positive result of $ALDH_1$. Concerning Scoring and Intensity of ALDH marker in the current study, score 2 was the highest in Group A (61.1%), score 1 had the highest prevalence in Group B (72.2%) and equal distribution of score 1 and 2 noticed in Group C (50% for each), furthermore, intensity take in consideration and Moderate intensity of $ALDH_1$ marker was the most commonly noticed in group A and B (61.1% and 81.8% respectively), while weak intensity was the most prevalent in group C (66.7%). Finally, sensitivity was 53.8%, specificity = 35.3% and accuracy of $ALDH_1$ marker was 43.3%.

Slightly different results noticed in a study conducted in Australia (2015) when 142 patients with PCa included, about half of the study patients (48%) exhibited cytoplasmic staining for $ALDH_1$, in which marked differences in the intensity of malignant cells expressing $ALDH_1$, ranging from <5% up to more than 90% observed.[18] Furthermore, In BPH, ALDH1A1 protein was detectable in 3 of 65 specimens (4.6%). In the few positive BPH cases, ALDH1A1 protein expression was clearly restricted to a few cells in the basal layer of the epithelium, while, ALDH7A1 protein in BPH was uniformly expressed in both basal and luminal cells.[19] $ALDH_1$ A1also involved in detoxification and has been implicated in the emergence of drug resistance to chemotherapeutic agents.[20] Fluorescence based biochemical studies have also shown that $ALDH_1$ is a useful marker for prostate CSCs. Consistent with a role in prostate cancer, $ALDH_1$ rich prostate cancer cells can initiate and propagate prostate cancers when transplanted into mice while those low in $ALDH_1$ do not.[21]

5.2. Gleason Grading System of PCa

Half of specimens of PCa (Group A) was graded ≤ 7 by Gleason Grading System (53.3%). Its association with CSC marker result was taken in consideration and half (55.6%) of positive $ALDH_1$ marker results were significantly seen between grade 8 and grade 9 ($P=0.024$), the association of (ALDH1) with Gleason score ($P > 0.005$) did not reach statistical significance, In contrary to Australian study in 2015, authors noticed that CSC marker $ALDH_1$ is expressed significantly in prostate carcinoma and more likely so in cases with adverse pathological parameters, such as extraprostatic extension, lymphovascular invasion, other studies had shown similar significant correlations between ALDH1 and poor prognosticators as well as disease free survival, as shown by studies done in USA in 2010[22], Australia in 2011[23] and Switzerland in 2013.[24]

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